Prolonged impairment of short-chain fatty acid and L-isoleucine biosynthesis in gut microbiome in patients with COVID-19

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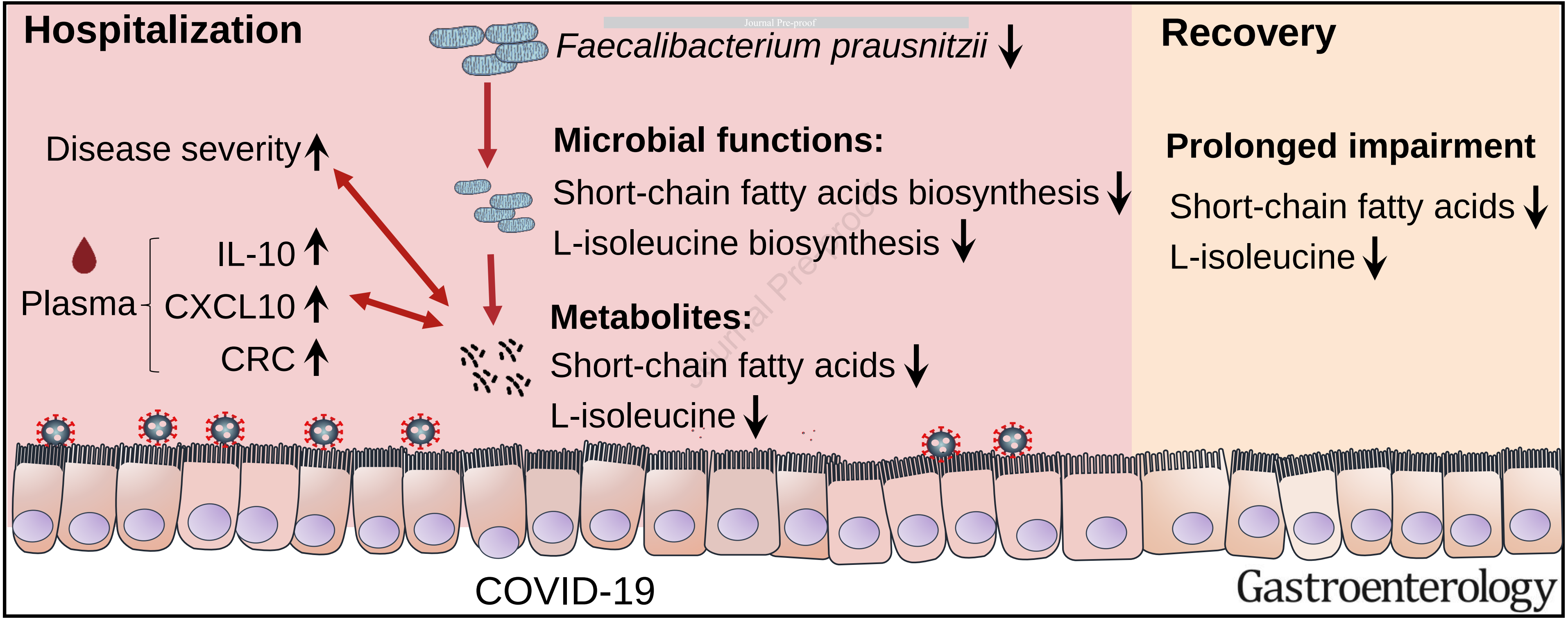
Hospitalization
Faecalibacterium prausnitzii ↓
Disease severity ↑
Microbial functions:
Short-chain fatty acids biosynthesis ↓
L-isoleucine biosynthesis ↓
Plasma
IL-10 ↑
CXCL10 ↑
CRC ↑
Metabolites:
Short-chain fatty acids ↓
L-isoleucine ↓
Recovery
Prolonged impairment
Short-chain fatty acids ↓
L-isoleucine ↓
COVID-19
Gastroenterology

Title：Prolonged impairment of short-chain fatty acid and L-isoleucine biosynthesis in gut microbiome in patients with COVID-19

Short title: Microbiome functional impairment in COVID-19

Fen Zhang[1,2,3,4#], **Yating Wan**[1,2,3,4#], Tao Zuo[1,2,3,4], Yun Kit Yeoh[1,5], Qin Liu[1,2,3,4], Lin Zhang[1,2,3,4,6], Hui Zhan[1,2,3,4], Wenqi Lu[1,2,3,4], Wenye Xu[1,2,3,4], Grace C.Y. Lui[4,7], Amy Y.L. Li[4], Chun Pan Cheung[1,2,3,4], Chun Kwok Wong[7], Paul K.S. Chan[1,2,5,8], Francis K.L. Chan[1,3,4], Siew C. Ng[1,2,3,4]*

1. Center for Gut Microbiota Research, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong, China
2. Li Ka Shing Institute of Health Science, The Chinese University of Hong Kong, Shatin, Hong Kong, China
3. State Key Laboratory for Digestive disease, Institute of Digestive Disease, The Chinese University of Hong Kong, Shatin, Hong Kong, China
4. Department of Medicine and Therapeutics, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong, China
5. Department of Microbiology, The Chinese University of Hong Kong, Shatin, Hong Kong, China
6. Department of Anaesthesia and Intensive Care and Peter Hung Pain Research Institute, The Chinese University of Hong Kong, Hong Kong, P.R. China;
7. Department of Chemical Pathology, The Chinese University of Hong Kong, Shatin, Hong Kong
8. Stanley Ho Centre for Emerging Infectious Diseases, The Chinese University of Hong Kong, Shatin, Hong Kong, China

#These authors contributed equally to this work: Fen Zhang, Yating Wan.

Correspondence*

Siew C Ng, Professor, Department of Medicine and Therapeutics, The Chinese University of Hong Kong, Tel: (852) 3505 3996; Fax: (852) 3505 3852, Email: siewchienng@cuhk.edu.hk

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Conflicts of interest

The authors disclose no conflicts.

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Author Contributions

FZ and YTW performed the experiments, data analyses and drafted the manuscript. TZ, YKY and LZ revised the manuscript and provided critical intellectual contribution. QL, HZ, WQL, WX assisted in experiments and metagenomics sequencing. GCYL recruited study subjects. AYLL and CPC collected the human specimens and data. FKLC provided critical comments. PKSC and CKW organized sample inventory and processing. SN designed and supervised the study.

Data availability

Raw sequence data generated for this study are available in the Sequence Read (Archive under BioProject accession PRJNA689961. https://www.ncbi.nlm.nih.gov/bioproject/ PRJNA689961)

ABSTRACT

Background & Aims: SARS-CoV-2 infection is associated with altered gut microbiota composition. Phylogenetic groups of gut bacteria involved in the metabolism of short chain fatty acids were depleted in SARS-CoV-2-infected patients**.** We aimed to characterize functional profile of gut microbiome in patients with COVID-19 before and after disease resolution.

Methods: We performed shotgun metagenomic sequencing on fecal samples from 66 antibiotics-naïve patients with COVID-19 and 70 non-COVID-19 controls. Serial fecal samples were collected (up to 6 times points) during hospitalization and beyond one month after discharge. We assessed gut microbial pathways in association with disease severity and blood inflammatory markers. We also determined changes of microbial functions in fecal samples before and after disease resolution and validated these functions using targeted analysis of fecal metabolites.

Results: Compared with non-COVID-19 controls, COVID-19 patients with severe/critical illness showed significant alterations in gut microbiome functionality (*P* < .001), characterized by impaired capacity of gut microbiome for short chain fatty acid (SCFA) and L-isoleucine biosynthesis and enhanced capacity for urea production. Impaired SCFA and L-isoleucine biosynthesis in gut microbiome persisted beyond 30 days after recovery in COVID-19 patients. Targeted analysis of fecal metabolites showed significantly lower fecal concentrations of SCFAs and L-isoleucine in COVID-19 patients before and after disease resolution. Lack of SCFA and L-isoleucine biosynthesis significantly correlated with disease severity and increased plasma concentrations of CXCL-10, NT-proBNP, C-reactive protein (CRP) (all *P* < .05).

Conclusions: Gut microbiome of COVID-19 patients displayed impaired capacity for SCFA and L-isoleucine biosynthesis which persisted even after disease resolution. These two microbial functions correlated with host immune response underscoring the importance of gut microbial functions in SARS-CoV-2 infection pathogenesis and outcome.

Keywords: Coronavirus; gut microbiome; microbial functions; SCFAs

Introduction

Coronavirus disease (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) primarily infects the respiratory system but also affects other organs including the gastrointestinal (GI) tract [1-4]. Recent studies have reported altered gut microbiome in SARS-CoV-2 infection[5-7], characterized by depletion of beneficial (butyrate-producing) bacteria, such as several genera from the Ruminococcaceae and Lachnospiraceae families and enrichment of opportunistic pathogens including *Streptococcus*, *Rothia*, *Veillonella*, and *Actinomyces*[8]. These alterations persisted even after recovery from COVID-19. Underrepresentation of several gut commensals with known immunomodulatory potential in fecal samples of COVID-10 patients including *Faecalibacterium prausnitzii*, *Eubacterium rectale* and *Bifidobacterium* reflects disease severity and dysfunctional host immune responses[9].

The gut microbiota is known to regulate host immune responses to respiratory viral infections[10-12]. Metabolites secreted by gut microbiome (such as tyrosine) could protect from influenza through the production of type I interferons and inflammasome-dependent cytokines by pulmonary cells[13, 14]. Whether microbial-derived metabolites also regulate host immune response to SARS-COV-2 infections remain unknown. Interestingly, an *in vitro* experiment demonstrated that butyrate downregulated genes essential for SARS-CoV-2 infection such as angiotensin-converting enzyme 2 (ACE2) and upregulated TLR (toll-like receptor) antiviral pathways in gut epithelial organoids[15].

Important microbial activities are a reflection of cumulative functions of the whole community of gut microbiota and the balance of the community and its output determines the net contribution to health or disease[16]. Deciphering the role of microbial-derived butyrate or other metabolites in SARS-CoV-2 pathogenesis and severity can shed light on our understanding of possible mechanistic links between gut microbiota and host defense against SARS-CoV-2.

In this study, we prospectively recruited 66 antibiotic treatment-naïve patients with COVID-19 and followed them up from hospital admission until 30 days after discharge. We characterized alterations and longitudinal dynamics of the functions of the gut microbiome in association with disease severity and immune response using metagenomic analysis. We further validated our findings using targeted metabolomics analysis to examine alterations in fecal microbial metabolites.

Methods

Subject recruitment and sample collection

This study was approved by the Clinical Research Ethics Committee (reference number 2020.076), and all patients provided written informed consent. As described in our previous study[17, 18], COVID-19 subjects were recruited at the Prince of Wales and United Christian Hospitals in Hong Kong. Inclusion criteria included: SARS-CoV-2 RT-PCR positive based on respiratory specimens, hospitalized, and had no probiotics, prebiotics and antibiotics use within 3 months before enrollment. Patients were classified into four severity groups based on symptoms as reported by Wu et al[11]. Briefly, patients were classified as mild if there were no radiographic indications of pneumonia, moderate if pneumonia with fever and respiratory tract symptoms were detected, severe if respiratory rate ≥30 breaths per minute, oxygen saturation ≤93% when breathing ambient air, or PaO2/FiO2 ≤300 mmHg, critical if respiratory failure requiring mechanical ventilation or organ failure requiring intensive care. Blood and stools from hospitalized patients were collected by hospital staff while discharged patients provided stools during follow-up visits. Samples were stored at -80 °C until processing. The pneumonia controls who were hospitalized with community-acquired pneumonia were recruited. Inclusion criteria included: SARS-CoV-2 RT-PCR negative based on respiratory specimens, hospitalized, and had no probiotics, prebiotics and antibiotics use within 3 months before enrollment. The non-COVID-19 subjects were recruited at the Prince of Wales Hospital in Hong Kong from the general population in 2019 before the pandemic as part of a microbiome survey. Inclusion criteria included: 18 years of age or older, had not used probiotics, prebiotics or antibiotics within 3 months before enrollment.

Stool DNA extraction

Detailed methods are described in Zuo et al[17]. Briefly, approximately 0.1g fecal sample was prewashed with 1 ml ddH_2O and pelleted by centrifugation at 13,000×g for 1 min. The fecal DNA was subsequently extracted from the pellet using Maxwell® RSC PureFood GMO and Authentication Kit (Promega, Madison, Wisconsin) following manufacturer's instructions.

Shotgun metagenomics sequencing and profiling

Sequencing libraries were prepared from extracted DNA using the Nextera DNA Flex Library Prep Kit (Illumina, California USA), and sequenced on an Illumina NovaSeq 6000 System at the Centre for Gut Microbiota Research, Chinese University of Hong Kong. An average of 32 ± 4.6 million reads (6G data) per sample were obtained.

Raw sequence reads were filtered and quality-trimmed using *Trimmomatic v0.36*[19] as follows: 1. Trimming low quality base (quality score < 20), 2. Removing reads shorter than 50bp, 3. Tracing and cutting off sequencing adapters. Contaminating human reads were filtering using *Kneaddata v0.7.3* (https://bitbucket.org/biobakery/kneaddata/wiki/Home, Reference database: GRCh38 p12) with default parameters.

Profiling of bacterial taxonomy and functional composition was extracted using *humann2 v0.11.1*[20] from metagenomes, which included taxonomic identification via *MetaPhlAn2* by mapping reads to clade-specific markers[21], annotation of species pangenomes through *Bowtie2 v2.3*[22] with reference to the ChocoPhlAn database, translated search of unmapped reads with *DIAMOND v2.0.4*[23] against the UniRef90 universal protein reference database[24] and pathway collection from the generated gene list with reference to the Metacyc database[25].

Plasma measurements

Laboratory results at admission including blood count test (platelet count, white cell count, neutrophil count) and the plasma concentrations of lactate dehydrogenase (LDH), C-reactive protein (CRP), albumin, hemoglobin, alkaline phosphatase (ALP) and aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin and creatinine, were extracted from the electronic medical records in Hong Kong Hospital Authority clinical management system. Concentrations of cytokines (IL10, IL12, IL1b, IL6 and TNF-α) and chemokines (CXCL8, CXCL10, CCL2) in patients at admission were measured using the MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel - Immunology Multiplex Assay (Merck Millipore, Massachusetts USA) on a Bio-Plex 200 System (Bio-Rad Laboratories, California, USA). Concentration of N-terminal proBNP (NT-proBNP) was measured using Human NT-proBNP ELISA kits (Abcam, Cambridge, UK).

Quantification of fecal metabolites

The quantification of fecal metabolites was performed by Metware Biotechnology Co., Ltd. (Wuhan, China). Short chain fatty acids (SCFA) including acetic, propionic, isobutyric, butyric, isovaleric, valeric, hexanoic acid were detected by GC-MS/MS analysis. Agilent 7890B gas chromatograph coupled to a 7000D mass spectrometer with a DB-5MS column (30 m length × 0.25 mm i.d. × 0.25 μm film thickness, J&W Scientific, USA) was used. Helium was used as carrier gas, at a flow rate of 1.2 mL/min. Injections were made in the splitless mode and the injection volume was 2 μL. The oven temperature was held at 90°C for 1 min, raised to 100°C at a rate of 25°C/min, raised to 150°C at a rate of 20°C/min and held at 150°C 0.6 min, further raised to 200°C at a rate of 25°C/min, held at 200°C 0.5 min. After running for 3 minutes, all samples were analyzed in multiple reaction monitoring mode. The temperature of injector inlet and transfer line were held at 200 °C and 230 °C, respectively.

L-isoleucine was detected by LC-MS analysis. LC-ESI-MS/MS system (UPLC, ExionLC AD, https://sciex.com.cn/; MS, QTRAP® 6500+ System, https://sciex.com/) was used for analysis. The analytical conditions for L-isoleucine were as follows, HPLC: column, Waters ACQUITY UPLC HSS T3 C18 (100 mm×2.1 mm i.d.,1.8 μm); solvent system, water with 0.05% formic acid (A), acetonitrile with 0.05% formic acid (B). The gradient was started at 5% B (0-10 min), increased to 95% B (10-11 min), and ramped back to 5% B (11-14 min); flow rate, 0.35 mL/min; temperature, 40°C; injection volume: 2 μL. The ESI source operation parameters were as follows: ion source, turbo spray; source temperature 550°C; ion spray voltage (IS) 5500 V (Positive), -4500 V (Negative); DP and CE for individual MRM transitions was done with further DP and CE optimization.

Statistical analysis

Data on the abundance of bacterial taxa and functionality were imported into R v3.5.1. Data on the functionality was normalized based on relative log expression (RLE) by Deseq2 (v1.26.0). Non-metric Multi-dimensional Scaling (NMDS) analysis were performed based on Bray-Curtis dissimilarities using vegan package (v2.5-3). Differential microbial functional pathway between COVID-19 patients and non-COVID-19 controls were identified using Deseq2 (v 1.26.0). Associations of microbial pathways with disease severity were identified using the multivariate analysis by linear models (MaAsLin) statistical frameworks implemented in the Huttenhower Lab Galaxy instance (http://huttenhower.sph.harvard.edu/galaxy/). Spearman correlations between microbial pathways, metabolites and the patients' plasma parameters were assessed by using cor and cor.test functions. Heat maps were generated using the pheatmap package (v1.0.10).

Results

Subjects' clinical characteristics

Sixty-six hospitalized patients with laboratory confirmed SARS-CoV-2 infection [29 males; mean ± standard error (SD) age of 42.6 ± 19.0 years], 70 age-, sex-matched controls (non-COVID-19 controls). Demographic, clinical characteristics and stool collection schedule were shown in Table 1 and Figure 1. Median duration of hospitalization for COVID-19 patients were 24 days (IQR, 4–46 days). Thirty-five patients were followed-up after discharge [median (IQR), 28 (18–41) days. All patients were antibiotic-naive and 35 patients (53.0%) received at least one anti-viral drugs. At admission, fifty-three patients (53.0%) presented with fever, eight patients (12.1%) had diarrhea, and 47 patients (71.2%) showed at least one respiratory symptom. Patients were stratified into critical (6.1%, n=4), severe (22.7%, n=15), moderate (24.2%, n=16) and mild (47.0%, n=31), according to the COVID-19 severity classification criteria[26].

Altered gut microbiome functional profile in patients with COVID-19

To understand how SARS-CoV-2 infection influences gut microbiome in antibiotics-naïve patients with COVID-19, ultra-deep metagenomic sequencing was performed on fecal samples from COVID-19 patients and controls, followed by taxonomic profiling of the fecal microbiome. We first investigated taxonomic composition of gut microbiota in COVID-19 patients with mild, moderate and severe/critical illness at baseline (the first stool sample collected after admission). The taxonomic composition in COVID-19 patients with severe/critical illness was significantly different from that of non-COVID-19 controls (Supplementary Figure 1A, PERMANOVA test, P < .001), and their Bray-Curtis dissimilarity to controls was significantly higher than that within non-COVID-19 individuals (Supplementary Figure 1B, Mann-Whitney U test, P < .05). The COVID-19 patients were primarily characterized by a depletion of *Bifidobacterium adolescentis*, *Ruminococcus_bromii*, *Faecalibacterium prausnitzii* and an enrichment of *Bacteroides ovatus*, *Bacteroides dorei*, *Bacteroides thetaiotaomicron*, compared with non-COVID-19 controls (MaAalin2 adjusting for age, gender and comorbidities, all adjust P <.05, Supplementary Table 1). Interestingly, decreased *Bifidobacterium adolescentis* and *Faecalibacterium prausnitzii* in patients with COVID-19 significantly associated with more severe symptom (MaAalin2 adjusting for age, gender and comorbidities, all adjust P <.02, Supplementary Table 2). These results suggest SARS-COV-2 infection is associated with altered composition of gut microbiome.

Since there was a difference in gut microbial composition between COVID-19 and non-COVID subjects, we examined whether these compositional differences translated into differences at the functional level by profiling the functional capacity of the gut microbiome, including the functional genes abundance and their corresponding pathways. We first investigated composition of microbial pathways of COVID-19 patients with mild, moderate and severe/critical illness at baseline (the first stool sample collected after admission). The composition of microbial pathways in COVID-19 patients with severe/critical illness was significantly different from that of non-COVID-19 controls (Figure 2A, PERMANOVA test, $P < .001$), and their Bray-Curtis dissimilarity to controls was significantly higher than that within non-COVID-19 individuals (Mann-Whitney U test, $P < .05$, Figure 2B). Among all host factors examined (age, gender, co-morbidities, COVID-19 disease severity, fecal SARS-CoV-2 viral load, antiviral drugs and diet), both SARS-CoV-2 infection and COVID-19 disease severity significantly impacted composition of microbial functional pathways, with COVID-19 disease severity showing the largest effect size ($R^2 = 0.073$, $P < .001$, PERMANOVA test, Figure 2C). Diet over the course of hospitalization (Supplementary Table 3) did not show significant effect in the variation of microbial functional pathway (PERMANOVA test, $R^2 = 0.01$, $P = 0.23$, Figure 2C). In addition, COVID-19 patients showed significantly lower richness of microbial pathways in their feces compared with non-COVID-19 controls (Figure 2D, $P < .05$). These results suggest that SARS-COV-2 infection is associated with altered functional profile of gut microbiome as well.

Depletion of beneficial microbial functions and its correlation with COVID-19 severity

We next interrogated which microbial functions primarily drive the difference in COVID-19 patients and non-COVID-19 controls. Nineteen microbial pathways were depleted, and nineteen pathways were enriched in fecal samples from COVID-19 patients at baseline compared to non-COVID-19 controls, which were determined by DESeq2 (all adjust P <.05) and adjusted for age and gender by MaAalin2 (all adjust P <.05). Seven out of nineteen depleted pathways in patient with COVID-19 were related to carbohydrate degradation indicating SARS-CoV-2 infection impaired gut microbiome's capacity to degrade carbohydrate. Bifidobacterium shunt pathway involved in biosynthesis of acetic acid showed greatest reduction (2.4-fold) while urea cycle pathway displayed greatest increase (2.3-fold), in COVID-19 patients compared with non-COVID-19 controls (Supplementary Table 4). These data suggest that COVID-19 patients demonstrated impaired capacity for short-chain fatty acid production and showed enhanced capacity for urea cycle. We then correlated the 38 differential pathways with COVID-19 disease severity via MaAsLin2 analysis adjusting for age, gender and comorbidities, 12 microbial pathways mainly related to sugar derivative degradation, L-isoleucine biosynthesis and purine nucleotide biosynthesis showed significantly negative correlation with COVID-19 severity (Table 2, FDR corrected p-value < .2), while 8 microbial pathways associated with carbohydrate biosynthesis, purine nucleotide biosynthesis, heme biosynthesis and peptidoglycan biosynthesis showed significantly positive correlation with COVID-19 severity (Table 2, FDR corrected p-value < .2). Sugar derivative degradation was associated with production of pyruvate which is the key metabolite for short chain fatty acid fermentation[27, 28]. L-isoleucine are crucial mediator in the microbiota-host crosstalk and play important roles in regulating host innate and adaptive immunity[29-31]. Alterations in the microbial functions in the gut of COVID-19 patients may have a significant impact in host physiology and functions.

To determine whether hospitalization may contribute to changes of gut microbiome, we have also included patients hospitalized with community-acquired pneumonia but were negative for COVID-19 (Supplementary Table 5). We found that patients with COVID-19 had significantly different gut microbiome function compared with pneumonia cases (Supplementary Figure 3, P = .004, PERMANOVA test). Among of thirty-nine enriched or depleted microbial pathways identified by comparing COVID-19 and non-COVID-19 controls (Supplementary Table 4). Twenty-one (51%) were also different between patients with COVID-19 and pneumonia cases including pathways associated with fecal short-chain fatty acid synthesis and isoleucine production (Supplementary Table 6, FDR corrected p-value < .2), These data suggest that alterations of gut microbiome functions in patients with COVID-19 is unlikely the result of hospitalization.

Correlations between microbial functions of SCFA and L-isoleucine biosynthesis and plasma measurements

SARS-CoV-2 infection could induce dysfunctional immune responses and consequently cytokine storm syndrome in a subset of COVID-19 patients leading to more severe disease outcomes[32]. Here, we found increased plasma levels of NT-proB-type natriuretic peptide (NT-proBNP), IL10, CXCL10, lactate dehydrogenase (LDH), C-reactive protein (CRP), Alanine aminotransferase (ALT) and decreased levels of platelet count (PLT), albumin and hemoglobin (MaAsLin2, FDR corrected p-value < .05) significantly associated with more severe symptoms in COVID-19 patients (Supplementary Table 7), suggesting SARS-CoV-2 infection could induce those changes consequently leading to more severe disease outcomes. We then link these changes in blood measurements (CXCL-10, IL-10, NT-proBNP, LDH, CRP, ATL, PLT and albumin) to the findings of microbial pathways (Table 2) to assess whether microbial functions play a role in dysregulation of the immune response. Four pathways (D-galacturonate degradation I, superpathway of hexuronide and hexuronate degradation, superpathway of β-D-glucuronosides degradation, and 4-deoxy-L-threo-hex-4-enopyranuronate degradation) involved in SCFA production significantly negatively correlated with NT-proBNP (Rho=-0.41, -0.48, -0.48, -0.47 respectively, Figure 3). Superpathway of L-isoleucine biosynthesis I negatively correlated with plasma levels of CXCL-10 (Rho=-0.35) and CRP (Rho=-0.35) (Figure 3). NT-proBNP is heart failure marker which was associated with more adverse clinical outcomes in patients with COVID-19 and increased significantly during the course of hospitalization in those who ultimately died[33]. CXCL10 is a pro-inflammatory chemokine known to be associated with poor outcome in COVID-19[34]. CRP levels in plasma increased in response to inflammation[35, 36]. These pathways may be involved in preventing overaggressive inflammation in COVID-19. In contrast, urea cycle pathway exhibited positive correlation with CXCL-10 (Rho=0.40), and heme biosynthesis II pathway showed negative correlation with PLT (Rho=-0.40) (Figure 3). Low platelet count was associated with increased risk of severe disease and mortality in COVID-19[37]. Hence, overexpression of these two pathways may be associated with more dysfunctional immune responses. Altogether, our data highlight that gut microbiome might functionally calibrate host immunity against SARS-CoV-2 infection thereby affecting COVID-19 severity.

Prolonged impairment of microbial functions of SCFA and L-isoleucine biosynthesis after recovery of COVID-19

To explore whether gut microbiome and their functions was restored in patients after recovery, fecal samples after discharge (post-discharge days, median (IQR), 28 (18–41) days) were collected from 35 patients (15 mild,17 moderate and 13 severe) (Figure 1). The patients with severe/critical illness still displayed a significantly different microbiome composition and functional capacity from that of non-COVID-19 controls after disease resolution (Supplementary Figure 2A, B and Figure 4A, B), although the richness of microbial pathway increased to a comparable level with non-COVID-19 controls (Figure 4C). We then investigated whether differential taxa (showed in Supplementary Table 1) or microbial functional pathways (showed in Table 2) were restored in patients with COVID-19 after disease resolution. Seven bacteria taxa including *Bifidobacterium adolescentis*, *Ruminococcus bromii*, *Faecalibacterium prausnitzii* which were depleted in patients' baseline samples, showed sustainable lower abundance after discharge when compared with non-COVID-19 controls. While *Bacteroides thetaiotaomicron* and *Bacteroides caccae* enriched at baseline still displayed higher abundance in patients with COVID-19 than non-COVID-19 controls even after disease resolution (Supplementary Figure 2C). In terms of microbial functions, all overrepresented pathways in severe patients' baseline samples returned to comparable level with non-COVID-19 controls after discharge. In contrast, nine out of 11 pathways, which were involved in SCFA and L-isoleucine biosynthesis, showed persistent depletion in severe patients after disease resolution (Figure 4D). These data suggest that gut microbiome functionality in patients persistently impaired after COVID-19 resolution.

Reduced fecal concentrations of SCFAs and L-isoleucine in COVID-19

Fecal metabolome provides a functional readout of microbial activity and can be used as a medium for mediating host-microbiome interaction[38]. Based on targeted analysis of fecal metabolites in COVID-19 patients and controls, we found that the changes of gut microbial functions were consistent with alterations of gut microbial metabolites. Several pathways associated with production of SCFAs and L-isoleucine were depleted in patients with COVID-19, especially in those severe/critical patients (Supplementary Table 4 and Table 2). We found fecal concentrations of SCFAs, including acetic acid, propionic acid, butyric acid, valeric acid and caproic acid, were significantly lower in COIVD-19 patients with severe/critical illness at baseline (the first stool sample collected after admission) than that in non-COVID-19 controls (Figure 6A-G). The concentrations of L-isoleucine in COVID-19 patients were lower than that in controls as well (P <.05, Figure 6H). These results further demonstrated SARS-CoV-2 infection may be associated with impaired capacity of gut microbiome for SCFAs production and L-isoleucine biosynthesis. Intriguingly, beyond 30 days after disease resolution, patients who had severe/critical illness still showed a significant lower concentration of butyric acid, valeric acid, caproic acid and L-isoleucine, supporting SARS-CoV-2 infection may cause long-lasting effect on gut microbiome function. In addition, we found fecal butyrate level in patients with COVID-19 showed significantly positive correlation with plasma IL-10, CXCL-10 and CRP, and negative correlation with albumin (all P < .05, Supplementary Figure 4). L-isoleucine positively correlated with CXCL-10 and negatively correlated with PLT (all P < .05, Supplementary Figure 5). These results suggest that microbiota-derived butyrate and L-isoleucine may be involved in preventing overaggressive inflammation in COVID-19.

We further examined which bacterial species contributed most to the changes of these microbial pathways. None of the pathways were dominated by any single species, suggesting that alterations of these pathways were likely accounted by a community-level shift in their functions (Supplementary Figure 5). Notably, the bacterium *Faecalibacterium prausnitzii* was a primary contributor to SCFA-producing pathways (D-galacturonate degradation I, superpathway of hexuronide and hexuronate degradation, superpathway of β-D-glucuronosides degradation) (Supplementary Figure 5A-D) and L-isoleucine-producing pathways (L-isoleucine biosynthesis I and L-isoleucine biosynthesis III) (Supplementary Figure 5 E and F), implicating *F. prausnitzii* may have a beneficial role in combating SARS-CoV-2 infection.

Discussion

Previous studies identified depleted bacterial taxa including *Eubacterium*, *Faecalibacterium*, *Roseburia*, *Bifidobacterium* and Lachnospiraceae in patients with COVID-19 compared with controls and inferred that these bacteria may have a functional role in the treatment of COVID-19 [6, 39, 40]. However mechanistic insights of such findings were limited until recently. To the best of our knowledge, this is the first study to delineate the functional potential and metabolic output of the entire gut microbiome community in COVID-19 which helps support development of microbiota-based therapy for COVID-19.

SCFAs, including butyrate, along with propionate and acetate, can exert anti-inflammatory effects through activating anti-inflammatory immune cells and inhibiting inflammatory signaling pathways[41]. In addition, butyrate can maintain integrity of gut barrier to prevent translocations and circulation of gut endotoxins and bacteria, thus reducing systemic inflammatory responses[42]. Recently, butyrate was found to protect host from viral infection via downregulating genes essential for SARS-CoV-2 infection such as angiotensin-converting enzyme 2 (ACE2) and upregulating TLR (toll-like receptor) antiviral pathways in gut epithelial organoids models[15]. In this study, we provided direct evidence that SCFAs depletion was associated with severe COVID-19 by fecal metabolite measurements, and demonstrated that decreased fecal butyrate level was associated with increased plasma levels of pro-inflammatory cytokine IL-10 and chemokine CXCL-10 which highlights the importance of SCFAs in COVID-19 pathogenesis and disease severity. Supplementation with SCFAs or probiotics-producing SCFA may have potential for improvement of disease outcome although this hypothesis requires further confirmation. Notably, impaired capacity for SCFAs biosynthesis and deficient fecal metabolite SCFAs in patients with COVD-19 persisted beyond 30 days after disease resolution which may cause the long-term health complications due to the importance of SCFA in host immunity and metabolism.

The gut microbiota also play a crucial role in manipulating the amino acid pool and profile over the course of amino acid digestion and absorption, thereby mediating the physiological aspects of the host[43]. We for the first time found that gut microbiota of COVID-19 patients had an attenuated capacity for L-isoleucine biosynthesis and they had lower fecal concentrations of L-isoleucine compared with controls. Recent studies have shown that L-isoleucine may induce expression of host defense peptides (i.e., β-defensins) that can regulate host innate and adaptive immunity and alleviates detrimental effect of pathogens on humans and animals[30, 31]. In line with that, we found that both L-isoleucine biosynthesis I pathway and fecal L-isoleucine displayed negative correlation with disease severity and a pro-inflammatory chemokine (CXCL-10). These results collectively support that L-isoleucine produced by gut microbiota may alleviate severity of COVID-19 by modulating the immune response of the host to SARS-COV-2 infection. In addition, L-isoleucine is one of branched chain amino acids (BCAAs) considered as nutritional supplements to improve central and muscular fatigue by increasing serum concentration of fatigue substances (lactate, ammonia and 5-HT), energy metabolites (glucose and free fatty acids) and muscle soreness substances (LDH and CK)[44, 45]. Interestingly, we observed that patients after recovery still have persistently impaired L-isoleucine biosynthesis and deficient fecal metabolite L-isoleucine. This may in part explain why COVID-19 survivors reported persistent symptoms of fatigue and muscle weakness[46]. Notably, we found the depletion of *Faecalibacterium prausnitzii* primarily contribute to the impaired capacity for SCFA and

L-isoleucine biosynthesis in patients with COVID-19. Beyond a depletion of *F. prausnitzii* reported by previous study, we provide possible modes of action that *F. prausnitzii* contribute to disease progression, underscoring the important role of this bacterium in COVID-19.

Furthermore, gut-microbiota-dependent urea metabolism are known to correlate with host urea balance and is implicated in disease progression[47]. We found that gut microbiome related urea cycle was enriched in COVID-19 samples and positively correlated with disease severity. Shen at al also found that COVID-19 patients had higher serum concentrations of urea than non-COVID-19 cases[48]. Altered gut microbiome may be associated with disruption of urea cycle functions during COVID-19 infection. However, how disruption of urea cycle impacts disease outcomes needs further investigation.

This study has several strengths. Subjects were all naïve to antibiotics (which is known to affect the microbiome) and followed up until after discharge with serial stool sample collection. The particularly striking finding was those distinct characteristics in a person's gut microbial functions (ie impaired SCFA biosynthesis) persisted after viral clearance and it is possible that these changes could contribute to the symptoms of so-called 'Long-COVID' including fatigue and muscle weakness. Although this remains speculative it demands further investigation. As hospital diet may contribute to the alteration of microbiome functions. We collected dietary record of patients over the course of hospitalization, and found the hospital diet had no significant effect on the variations of the microbiome functions in patients during hospitalization. Collectively, the change of diet is unlikely to account for our findings. This study has some limitations. We unfortunately did not capture the diet of non-COVD-19 controls or that of COVID-19 patients before disease onset, which precludes us to investigate the virtual effect of diet in gut microbiome alteration between COVID-19 and non-COVID-19, although the hospital diet was aligned with the habitual diets commonly consumed by Hong Kong Chinese. Moreover, it is an observational study and cannot indicate whether variations in gut microbial functions is determining COVID-19 severity or whether the virus itself has caused this variation. Further studies are required to determine whether these changes to the gut microbiome functions directly affect the severity of COVID-19 in patients, or whether they are simply a consequence of the effects of the infection on the gut and the immune system. Future mechanistic studies are warranted to confirm the impact of SCFA and L-isoleucine on disease severity and their role in preventing viral infection. It is also not certain whether similar changes are observed in patients in other geographical regions.

In conclusion, this study showed perturbations of gut microbial functions including decreased SCAF and L-isoleucine biosynthesis and increased urea production in COVID-19. This highlights a potential link between impaired gut microbiome functions and COVID-19 severity and paves the way to understand role of gut microbiome in disease onset and progression in COVID-19.

Figure Legends

Figure 1. Schematic diagram of stool sample collection and hospitalization duration in patients with COVID-19 (n=66). “CoV” denotes patient with COVID-19. “D0” denotes to when the patients reported illness onset.

Figure 2. Altered gut microbiome functional profile in patients with COVID-19 at baseline. A. The composition of microbial functional pathways in COVID-19 patients with mild, moderate and severe/critical illness and non-COVID-19 individuals, viewed by NMDS (Non-metric multidimensional scaling) plot based upon Bray-Curtis dissimilarities. The p value of the significance was determined by PERMANOVA analysis and was indicated as ***P <.001. B. The Bray-Curtis dissimilarity of COVID-19 patients with mild, moderate and severe/ critical illness to non-COVID-19 controls based on abundance of pathways. The p value of the significance was determined by Mann-Whitney U test, and was indicated as ***P < .001. C. The effect size of host factors on the composition of microbial pathways. The effect size and statistical significance was determined via PERMANOVA analysis. *, **, *** was indicated as P < .05, P < .01, P < .001 respectively. D. Richness of microbial pathways in COVID-19 patients with mild, moderate and severe/critical illness and non-COVID-19 individuals, evaluated based on Chao1 index. The statistical significance between COVID-19 patients with non-COVID-19 controls was determined by Mann-Whitney U test, and was indicated as $^{\#}P$ < .05.

Figure 3. Spearman correlations between microbial pathways and plasma measurements. Those pathways or plasma measurements which were significantly correlated with disease severity were plotted. Blue circles and positive values indicate positive correlations, and red circles and negative values indicate inverse correlations. The size and shading indicate the magnitude of the correlation, where darker shades showed higher correlations than lighter ones.

Figure 4. Prolonged impairment of microbial functions of SCFAs and L-isoleucine biosynthesis after recovery of COVID-19. A. The composition of microbial pathways in non-COVID-19 controls as well as COVID-19 patients at baseline and after discharge, viewed by NMDS (Non-metric multidimensional scaling) plot based upon Bray-Curtis dissimilarities. B. The Bray-Curtis dissimilarity of COVID-19 patients at baseline and after discharge to non-COVID-19 controls. The p value of the significance was determined by Mann-Whitney U test and was indicated as $^*P < .05$. C. Richness of microbial pathways in non-COVID-19 controls as well as COVID-19 patients at baseline and after discharge, evaluated based on Chao1 index. $^{\#}P < .05$ indicate statistical significance for patients with COVID-19 versus non-COVID-19 controls, determined by Mann-Whitney U test. D. Heat map summarizing changes in gut microbiome functionality in patients with COVID-19 after discharge. The labels on right of Figure indicate microbial pathways. Pathways with higher abundances are shaded red, whereas those with low relative abundances are shaded blue.

Figure 5. The fecal concentration of acetic acid (A), propionic acid (B), butyric acid (C) valeric acid (D), hexanoic acid (E), isobutyric acid (F) isovaleric acid (G) and L-isoleucine (H) in COVID-19 patients and non-COVID-19 controls. The first stool sample collected after admission (baseline group) and the stool collected after discharge (post-recovery group) were used for the targeted metabolome analysis.

$^*P < .05$, $^{**}P < .01$ and $^{**}P < .001$ indicates statistical significance for mild, moderate and severe/critical patients versus non-COVID-19 controls, determined by Mann-Whitney U test.

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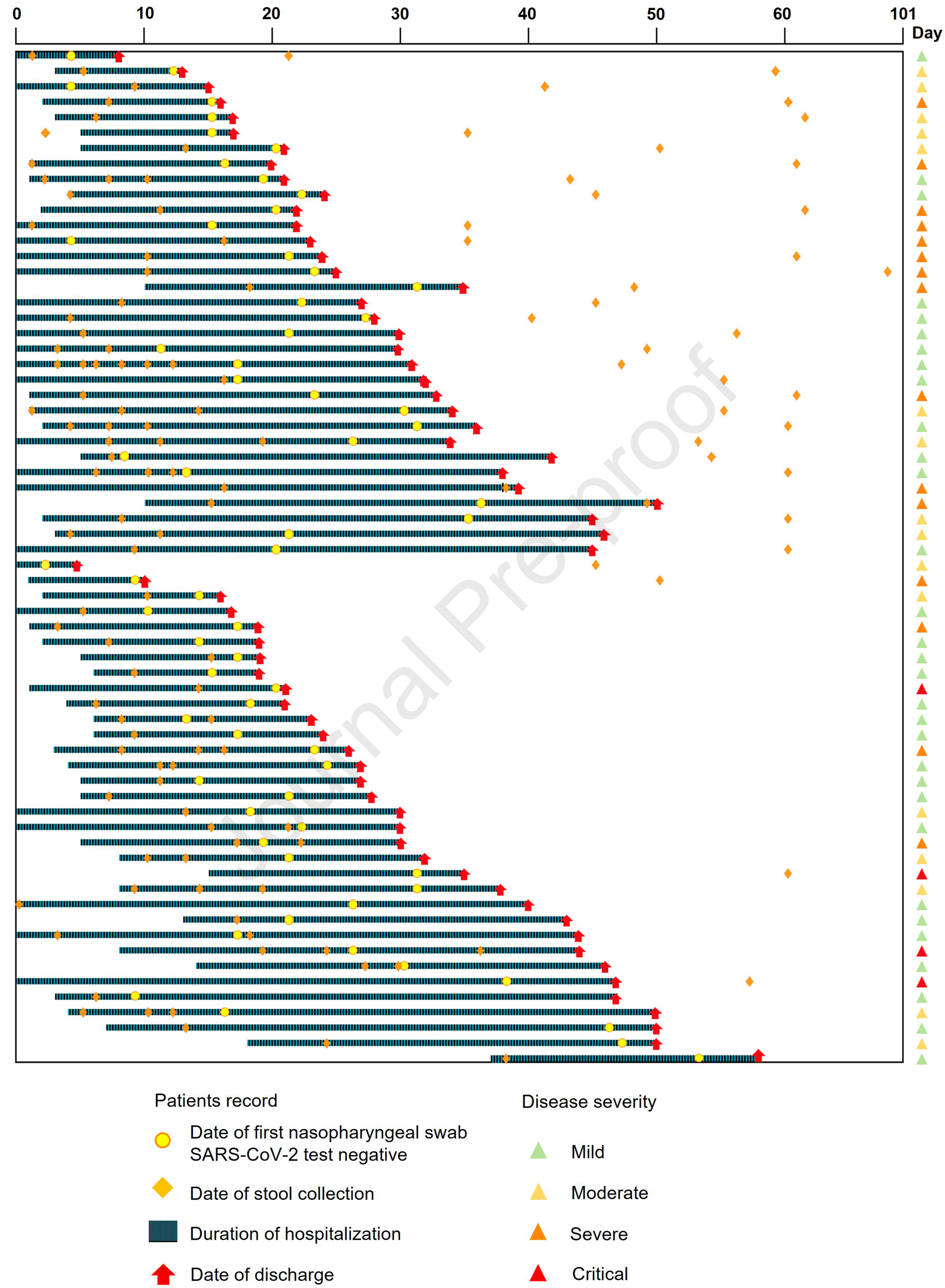
Table 1. Clinical characteristics of COVID-19 cases and non-COVID-19 controls

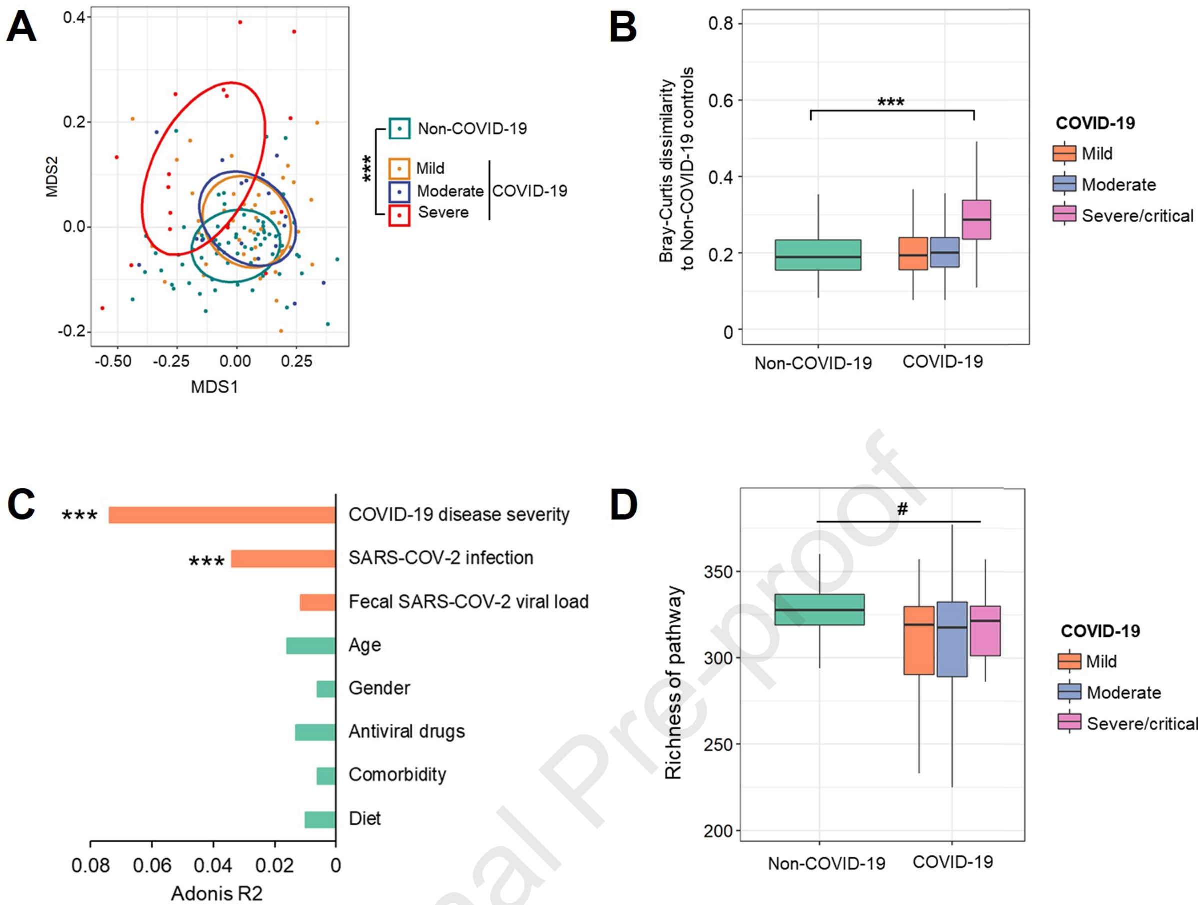
Characteristic	COVID-19 cases	Non-COVID-19 controls
Number of subjects	66	70
Male	29 (59.1%)	29 (41.0%)
Age, years (mean ± sd)	42.6 (± 19.0)	45.8 (±13.7)
Co-morbidities, n (%)	18 (27.3%)	20 (29.0%)
Hypertension	6 (9.1%)	10 (14.3%)
Hyperlipidemia	7 (10.6%)	0 (0.0%)
Heart disease	1 (1.5%)	0 (0.0%)
Eczema	1 (1.5%)	2 (2.9%)
Morbid obesity	0 (%)	0 (%)
HIV	2 (3.0%)	0 (0.0%)
Allergic rhinitis	2 (3.0%)	7 (10%)
Asthma	3 (4.5%)	2 (2.9%)
Gastric ulcer	0 (0.0%)	0 (0.0%)
Acid reflux disease	0 (0.0%)	0 (0.0%)
Bowel disease	0 (0.0%)	5 (7.1%)
Hemorrhoids	0 (0.0%)	4 (5.7%)
Diabetes	3 (4.5%)	0 (0.0%)
HBsAg	3 (4.5%)	0 (0.0%)
Pulmonary disease	0 (0.0%)	0 (0.0%)
Duration of hospitalization	24 (4-46)	
Symptoms at admission, n (%)		
Fever	35 (53.0%)	
Gastrointestinal symptoms		
Diarrhea	8 (12.1%)	
Respiratory symptoms	47 (71.2%)	
Cough	24 (36.4%)	
Sputum	12 (18.2%)	
Rhinorrhea (runny nose)	14 (21.2%)	
Shortness of breath (dyspnea)	9 (13.6%)	
Blood result		
Lymphocyte counts ($x10^9$/L, normal range 1.1-2.9, median (IQR))	1.2 = (1.0, 1.7)	
Antiviral therapy, n (%)		
Kaletra	30 (45.5%)	
Oseltamivir	2 (3.0%)	
Ribavirin	14 (21.2%)	
Interferon Beta-1B	23 (34.8%)	
Remdesivir	9 (13.6%)	
Death, n (%)	1 (1.5%)	

Table 2. Microbial pathways significantly correlated with disease severity

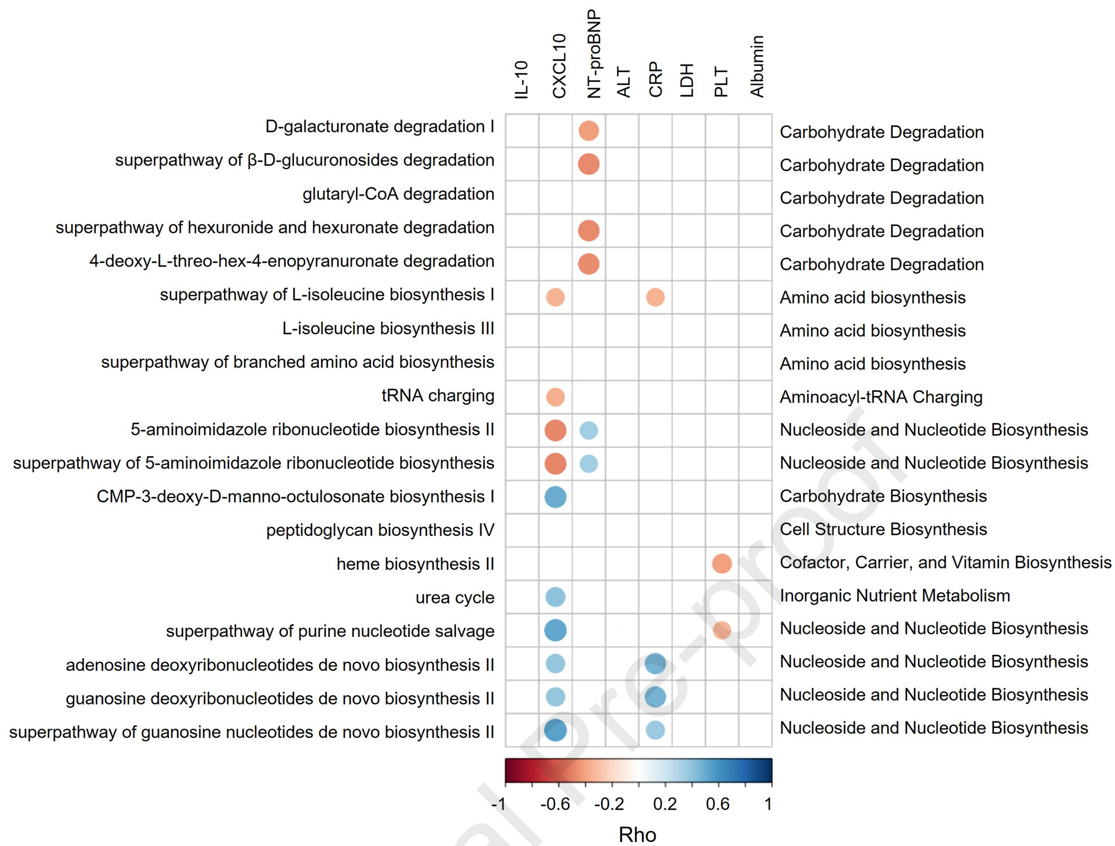
Pathway	Subclass	Coefficient	FDR corrected p-value	
PWY-5177: glutaryl-CoA degradation	glutaryl-CoA degradation	-267.0167	0.0143	Negative correlation with disease severity
PWY-3001: superpathway of L-isoleucine biosynthesis I	L-isoleucine biosynthesis	-557.1294	0.0387	
TRNA-CHARGING-PWY: tRNA charging	Aminoacyl-tRNA Charging	-602.3432	0.0387	
PWY-5103: L-isoleucine biosynthesis III	L-isoleucine biosynthesis	-654.6095	0.0387	
BRANCHED-CHAIN-AA-SYN-PWY: superpathway of branched amino acid biosynthesis	Branched amino acid biosynthesis	-647.6653	0.0387	
PWY-6122: 5-aminoimidazole ribonucleotide biosynthesis II	Purine Nucleotide Biosynthesis	-849.5535	0.0429	
PWY-6277: superpathway of 5-aminoimidazole ribonucleotide biosynthesis	Purine Nucleotide Biosynthesis	-849.5535	0.0429	
GALACTUROCAT-PWY: D-galacturonate degradation I	Sugar derivative degradation	-236.7136	0.0611	
GALACT-GLUCUROCAT-PWY: superpathway of hexuronide and hexuronate degradation	Sugar derivative degradation	-204.1731	0.0885	
GLUCUROCAT-PWY: superpathway of β-D-glucuronosides degradation	Sugar derivative degradation	-199.9632	0.0885	
PWY-6507: 4-deoxy-L-threo-hex-4-enopyranuronate degradation	Sugar derivative degradation	-174.8413	0.1631	
PWY-1269: CMP-3-deoxy-D-manno-octulosonate biosynthesis I	Sugar Nucleotide Biosynthesis	727.2037	0.0043	Positive correlation with disease severity
PWY-7220: adenosine deoxyribonucleotides de novo biosynthesis II	Purine Nucleotide Biosynthesis	757.6133	0.0043	
PWY-7222: guanosine deoxyribonucleotides de novo biosynthesis II	Purine Nucleotide Biosynthesis	757.6133	0.0043	
PWY-4984: urea cycle	Nitrogen Compound Metabolism	337.4701	0.0043	
HEMESYN2-PWY: heme biosynthesis II (anaerobic)	Heme biosynthesis	519.5936	0.0043	
PWY-6125: superpathway of guanosine nucleotides de novo biosynthesis II	Purine Nucleotide Biosynthesis	818.4742	0.0048	
PWY66-409: superpathway of purine nucleotide salvage	Purine Nucleotide Biosynthesis	476.2132	0.0170	
PWY-6471: peptidoglycan biosynthesis IV (Enterococcus faecium)	Peptidoglycan Biosynthesis	205.5374	0.0515	

The correlations between pathways and disease severity were determined by MaAsLin2, adjusting for age, gender and comorbidities. Only the pathways with false discovery rate (FDR) corrected p-value <0.2 were shown.


0
10
20
30
40
50
60
101
Day
Patients record
Date of first nasopharyngeal swab SARS-CoV-2 test negative
Date of stool collection
Duration of hospitalization
Date of discharge
Disease severity
Mild
Moderate
Severe
Critical



A
0.4
0.2
0.0
-0.2
MDS2
-0.50
-0.25
0.00
0.25
MDS1
Non-COVID-19
Mild
Moderate
Severe
COVID-19
***
B
0.8
0.6
0.4
0.2
0
Bray-Curtis dissimilarity to Non-COVID-19 controls
***
Non-COVID-19
COVID-19
COVID-19
Mild
Moderate
Severe/critical
C
***
***
COVID-19 disease severity
SARS-COV-2 infection
Fecal SARS-COV-2 viral load
Age
Gender
Antiviral drugs
Comorbidity
Diet
0.08
0.06
0.04
0.02
0
Adonis R2
D
#
350
300
250
200
Richness of pathway
Non-COVID-19
COVID-19
COVID-19
Mild
Moderate
Severe/critical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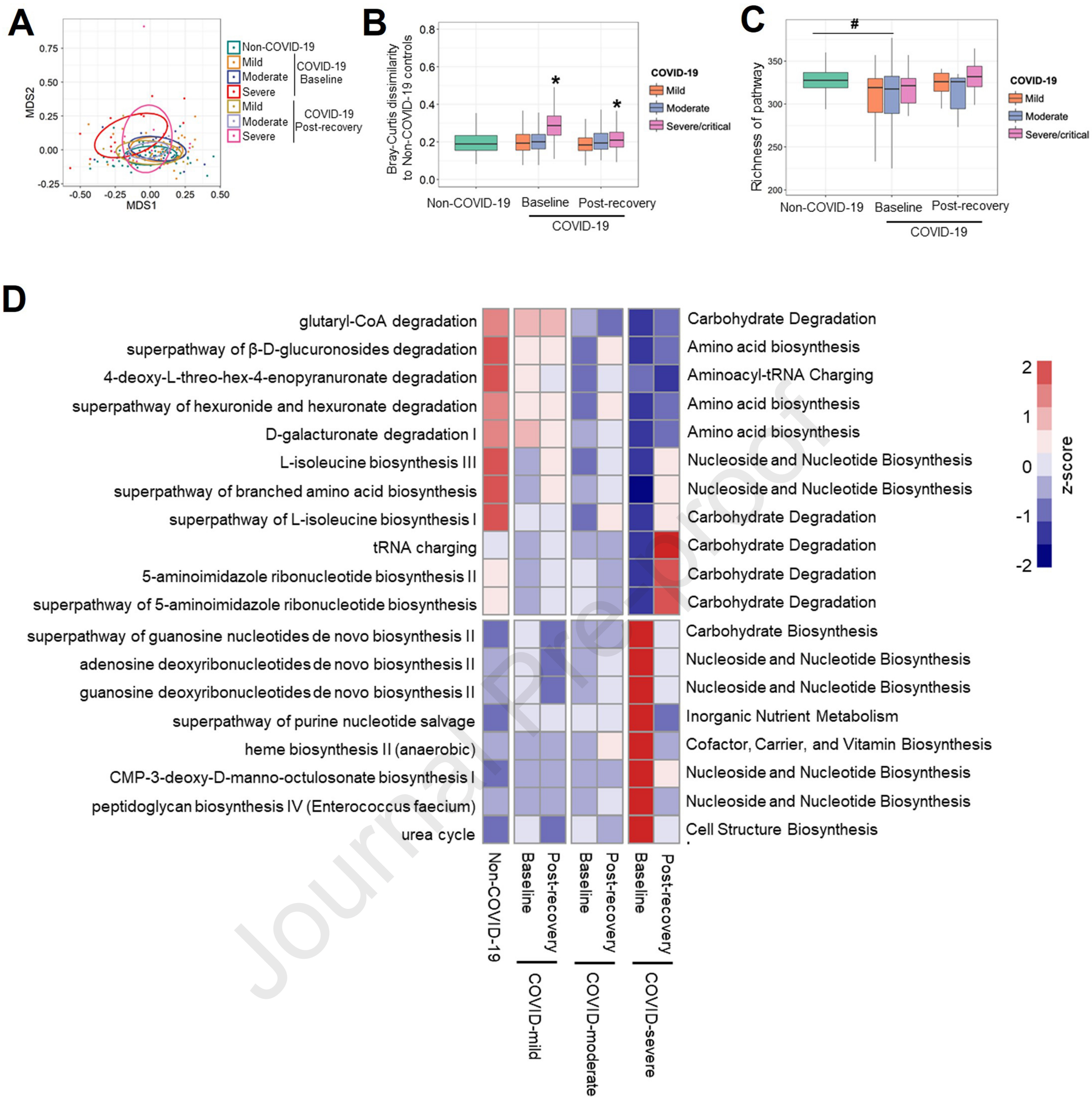
CXCL10
NT-proBNP
ALT
CRP
LDH
PLT
Albumin
D-galacturonate degradation I
superpathway of β-D-glucuronosides degradation
glutaryl-CoA degradation
superpathway of hexuronide and hexuronate degradation
4-deoxy-L-threo-hex-4-enopyranuronate degradation
superpathway of L-isoleucine biosynthesis I
L-isoleucine biosynthesis III
superpathway of branched amino acid biosynthesis
tRNA charging
5-aminoimidazole ribonucleotide biosynthesis II
superpathway of 5-aminoimidazole ribonucleotide biosynthesis
CMP-3-deoxy-D-manno-octulosonate biosynthesis I
peptidoglycan biosynthesis IV
heme biosynthesis II
urea cycle
superpathway of purine nucleotide salvage
adenosine deoxyribonucleotides de novo biosynthesis II
guanosine deoxyribonucleotides de novo biosynthesis II
superpathway of guanosine nucleotides de novo biosynthesis II
Carbohydrate Degradation
Carbohydrate Degradation
Carbohydrate Degradation
Carbohydrate Degradation
Carbohydrate Degradation
Amino acid biosynthesis
Amino acid biosynthesis
Amino acid biosynthesis
Aminoacyl-tRNA Charging
Nucleoside and Nucleotide Biosynthesis
Nucleoside and Nucleotide Biosynthesis
Carbohydrate Biosynthesis
Cell Structure Biosynthesis
Cofactor, Carrier, and Vitamin Biosynthesis
Inorganic Nutrient Metabolism
Nucleoside and Nucleotide Biosynthesis
Nucleoside and Nucleotide Biosynthesis
Nucleoside and Nucleotide Biosynthesis
Nucleoside and Nucleotide Biosynthesis
-1
-0.6
-0.2
0.2
0.6
1
Rho



A
MDS1
MDS2
Non-COVID-19
Mild
Moderate
Severe
COVID-19 Baseline
Mild
Moderate
Severe
COVID-19 Post-recovery
B
Bray-Curtis dissimilarity to Non-COVID-19 controls
Non-COVID-19
Baseline
Post-recovery
COVID-19
COVID-19
Mild
Moderate
Severe/critical
C
Richness of pathway
Non-COVID-19
Baseline
Post-recovery
COVID-19
Mild
Moderate
Severe/critical
D
glutaryl-CoA degradation
superpathway of β-D-glucuronosides degradation
4-deoxy-L-threo-hex-4-enopyranuronate degradation
superpathway of hexuronide and hexuronate degradation
D-galacturonate degradation I
L-isoleucine biosynthesis III
superpathway of branched amino acid biosynthesis
superpathway of L-isoleucine biosynthesis I
tRNA charging
5-aminoimidazole ribonucleotide biosynthesis II
superpathway of 5-aminoimidazole ribonucleotide biosynthesis
superpathway of guanosine nucleotides de novo biosynthesis II
adenosine deoxyribonucleotides de novo biosynthesis II
guanosine deoxyribonucleotides de novo biosynthesis II
superpathway of purine nucleotide salvage
heme biosynthesis II (anaerobic)
CMP-3-deoxy-D-manno-octulosonate biosynthesis I
peptidoglycan biosynthesis IV (Enterococcus faecium)
urea cycle
Carbohydrate Degradation
Amino acid biosynthesis
Aminoacyl-tRNA Charging
Amino acid biosynthesis
Amino acid biosynthesis
Nucleoside and Nucleotide Biosynthesis
Nucleoside and Nucleotide Biosynthesis
Carbohydrate Degradation
Carbohydrate Degradation
Carbohydrate Degradation
Carbohydrate Degradation
Carbohydrate Biosynthesis
Nucleoside and Nucleotide Biosynthesis
Nucleoside and Nucleotide Biosynthesis
Inorganic Nutrient Metabolism
Cofactor, Carrier, and Vitamin Biosynthesis
Nucleoside and Nucleotide Biosynthesis
Nucleoside and Nucleotide Biosynthesis
Cell Structure Biosynthesis
z-score
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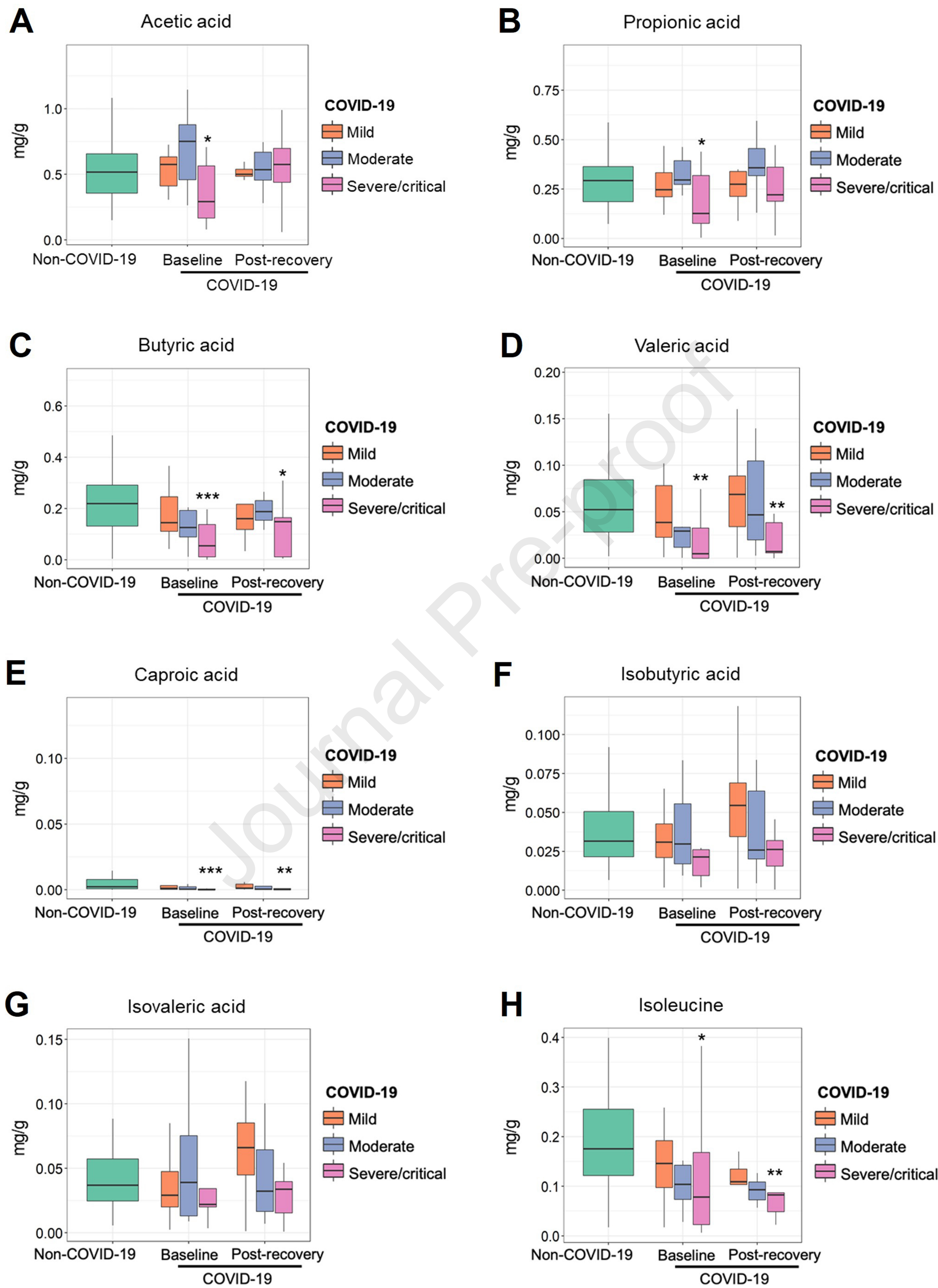
Baseline
Post-recovery
COVID-mild
Baseline
Post-recovery
COVID-moderate
Baseline
Post-recovery
COVID-severe


A
Acetic acid
mg/g
1.0
0.5
0.0
*
Non-COVID-19
Baseline
Post-recovery
COVID-19
COVID-19
Mild
Moderate
Severe/critical
B
Propionic acid
0.75
0.50
0.25
0.00
C
Butyric acid
0.6
0.4
0.2
***
D
Valeric acid
0.20
0.15
0.10
0.05
**
E
Caproic acid
0.10
0.05
F
Isobutyric acid
0.100
0.075
0.050
0.025
0.000
G
Isovaleric acid
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H
Isoleucine
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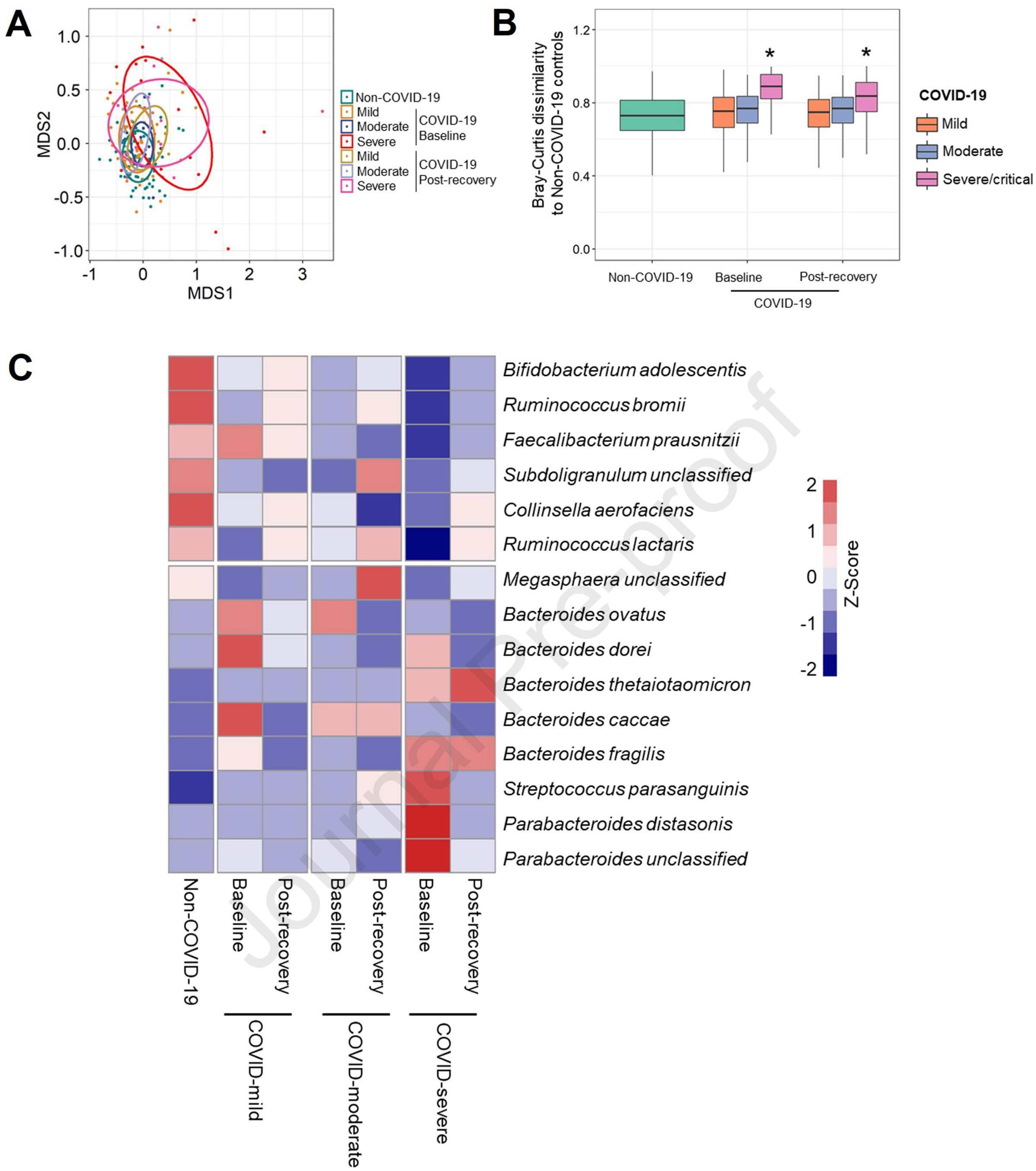
0.3
0.2
0.1


A
1.0
0.5
0.0
-0.5
-1.0
MDS2
-1
0
1
2
3
MDS1
Non-COVID-19
Mild
Moderate
Severe
COVID-19 Baseline
Mild
Moderate
Severe
COVID-19 Post-recovery
B
Bray-Curtis dissimilarity to Non-COVID-19 controls
1.2
0.8
0.4
0.0
Non-COVID-19
Baseline
Post-recovery
COVID-19
COVID-19
Mild
Moderate
Severe/critical
C
Bifidobacterium adolescentis
Ruminococcus bromii
Faecalibacterium prausnitzii
Subdoligranulum unclassified
Collinsella aerofaciens
Ruminococcus lactaris
Megasphaera unclassified
Bacteroides ovatus
Bacteroides dorei
Bacteroides thetaiotaomicron
Bacteroides caccae
Bacteroides fragilis
Streptococcus parasanguinis
Parabacteroides distasonis
Parabacteroides unclassified
2
1
0
-1
-2
Z-Score
Non-COVID-19
Baseline
Post-recovery
COVID-mild
Baseline
Post-recovery
COVID-moderate
Baseline
Post-recovery
COVID-severe

Background and Context

SARS-CoV-2 infection is associated with altered gut microbiota composition. The authors characterized functional profile of gut microbiome and examined fecal metabolites in patients COVID-19 before and after disease resolution.

New Findings

COVID-19 patients displayed impaired capacity for short-chain fatty acid (SCFA) and L-isoleucine biosynthesis in their gut microbiome which persisted after recovery and correlated with disease severity and host immune responses.

Limitations

It is an observational study without clear cause or consequence effect established. Further studies are required to determine whether these changes to the gut microbiome functions directly affect COVID-19 severity.

Impact

These findings indicate perturbations of gut microbial functions including decreased SCAF and L-isoleucine biosynthesis in COVID-19 before and after disease resolution. Strategies to supplement SCAF or L-isoleucine might be developed to improve disease outcome.

Lay Summary: Gut microbiome of COVID-19 patients displayed impaired capacity for SCFAs and L-isoleucine biosynthesis. Further studies are needed to understand how impaired gut microbial functions are involved in disease outcome.